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GLYCOPINION MINI-REVIEW

Will the transgenic mouse serve as a Rosetta Stone to glycoconjugate function?

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The overwhelming diversity of oligosaccharide structures on glycoproteins and glycolipids is both the most fascinating and the most frustrating aspect of glycobiology. Moreover, a single protein may be variably glycosylated and thereby represented by multiple glycoforms. As envisioned, many modifications may serve no useful function while others are likely to be essential [1]; hence, experimental approaches to understand the biological basis for such complexity can be difficult to formulate. In a recent comprehensive review on oligosaccharide function [2], Varki concludes that oligosaccharides carry out a large number of biological roles and that 'while all theories are correct, exceptions to each can be found'. Although a common theme to oligosaccharide function may never appear, crucial biological information can be observed to reside within various glycoforms. Examples include the glycoform-dependent mechanism of selectin function in mediating haemopoietic cell extravasation during inflammatory responses [3] and the clearance of particular glycoforms from serum by various glycoform-specific receptors [4–6]. Together, studies of glycosyltransferase biochemistry, naturally-occurring and experimentally-induced glycoform mutations, and the genetic basis for the production of glycoform complexity have allowed crucial steps in the biosynthesis of specific glycan structures to be reconstructed as they appear to occur in the endoplasmic reticulum and Golgi apparatus of intact cells [7]. With a significant foundation of biochemical knowledge achieved, genetic approaches are under way further to decipher the physiological roles encoded within the diverse and dynamic mammalian oligosaccharide repertoire.

Glycoform diversity is mediated by a glycosyltransferase gene superfamily with biologically unique members

The hypothesis that there is a separate glycosyltransferase for every glycosidic linkage (there are a few exceptions to this rule) and the diversity of oligosaccharide structures suggest that there are at least 100–200 different genes that comprise the glycosyltransferase superfamily. As implied, members representing distinct families, such as *N*-acetylglucosaminyltransferases, galactosyltransferases, sialyltransferases and fucosyltransferases, differ functionally in the saccharide unit transferred and the glycosidic linkage formed. The genes for more than 20 of these enzymes have been cloned to date [8–10]. Glycosyltransferases show exquisite substrate specificity for previously generated oligosaccharide structures. Moreover, the generation of multiple glycoforms (microheterogeneity) may result at least in part from changes in glycosyltransferase expression or activity during the synthesis of a particular glycoprotein. An understanding of the genetic control of temporally- and spatially-dependent glycoconjugate synthesis is at hand with the availability of cloned glycosyltransferase genes.

Different proteins may express identical oligosaccharide modifications, while different cells may express distinct glycoforms of an otherwise identical protein. The latter can occur by virtue of variations in glycosyltransferase expression profiles that alter the oligosaccharide repertoire of a given cell. Although the polypeptide backbone may impart limitations to oligosaccharide structures and bestow unique conformations essential for specific glycoform function, recent gene transfer experiments in COS cells have revealed that even immunoglobulin (Ig)-fusion proteins can function as E-selectin (ELAM-1) ligands upon co-transfection and expression of the α1,3-fucosyltransferase required for ligand synthesis (A. Aruffo, personal communication). It is also possible that high expression of relatively few glycoproteins may restrict oligosaccharide variation on the cell surface by effectively competing with other glycosyltransferase substrates. Examples may include the CD43 and CD45 molecules that appear to be major 'templates' for changing T-cell surface oligosaccharide diversity upon activation and malignant transformation [11–13]. Hence, relatively few substrates may exist for some glycosyltransferases, especially those that act at terminal positions in the

pathway of glycoform synthesis. Therefore, it can be theorized that biological information is contained within the oligosaccharide structures themselves and not necessarily by virtue of their association with any one particular peptide-based backbone.

Are phylogenic variations in glycoform diversity informative?

Although only a small portion of a phylogenetic analysis has been accomplished to date, the diversity of complex asparagine-(*N*)-linked glycans appears common only among higher eukaryotes. Specifically, complex *N*-glycans are found in vertebrates which exist within the Phylum *Chordata*, characterized by organisms with notochords, while lower eukaryotes including yeast and multi-cellular fungi do not appear to modify the processed mannose core in this manner [14, 15]. Additionally, studies of organisms within the *Insectae* class (contained within the largest Phylum – *Arthropoda*) have not at present yielded evidence for a diverse repertoire of complex *N*-glycans. However, some insect species may express certain complex *N*-linked glycoforms in embryonic development [16], and perhaps in response to stimuli that may be mimicked by cell culture conditions [17–20]. Nevertheless, complex glycoforms, at least those of the *N*-linked variety, are not crucial for life *per se*.

Glycoform diversity evolved following glycosyltransferase gene duplication, conversion and mutation that resulted in altered patterns of expression and changes in substrate specificity. Resulting variation in oligosaccharide diversity can be considered, at least in part, as a mechanism that evolved to allow complex organisms to adapt to the trials of natural selection in a changing and challenging environment. As long as the novel glycosyltransferase-generated glycoforms, as a whole, provided a selective advantage, these apparently pleiotropic modifications may have become prevalent among many species in phylogeny. Additionally, co-evolution of both glycosyltransferases and the proteins modified could have occurred. In simple organisms, relatively few glycosyltransferases may have each modified only one or a few molecular targets. Perhaps glycosyltransferase action imposes unique constraints on evolving protein structure and function, constraints that may be overly restrictive in less complex organisms with fewer genes.

Genetic variations in pathogens have likely played a role in the generation of mammalian glycosyltransferase diversity. In one fascinating possibility, a reduction in oligosaccharide diversity via α1,3-galactosyltransferase gene inactivation may have allowed an ancestor of the catarrhine group of primates, including *Homo Sapiens*, to survive in the presence of a virulent pathogen which gained the ability to invade host cells via recognition of specific glycoform determinants on the cell surface [21]. Survival may have depended upon the ability of Old World primates continually to generate an effective antibody-mediated immune response since high levels of 'anti-α1,3-linked Gal' antibodies are found in normal catarrhine serum. Evasion of infectious pathogens may be equally facilitated by addition to the repertoire of glycosyltransferases that might act to mask an otherwise fatal glycoform determinant in the host. Regardless of the role of pathogens in the acquired oligosaccharide diversity of multicellular organisms, millions of years of evolution have specifically layered distinct saccharide units in composing the present-day oligosaccharide repertoire in mammals. Characterizing phylogenic and experimentally-induced variations in the oligosaccharide repertoire is likely further to aid in the identification of glycoform-mediated biological processes.

Glycoform variants provide views of oligosaccharide function in metabolism and disease

The study of mutations has long been a classic approach to the elucidation of biological functions. Several human in-born errors involving defects in glycosylation have been found, thus providing connections between changes in oligosaccharide structure and disease. One of the earliest noted examples is Inclusion Cell (I-cell) Disease in which there is a defect in the synthesis of the mannose-6-phosphate signal required for targeting to the lysosome [22–23]. Other more recent examples are Congenital Dyserythropoietic Anaemia Type II (Hereditary Erythroblastic Multinuclearity with a Positive Acidified Serum Test, HEMPAS) [24–25] and Leukocyte Adhesion Deficiency Type II [26–27]. Additionally, a close correlation exists between cell surface carbohydrate structure and metastatic potential [28]. Since glycosyltransferase genes have become available only relatively recently, it is possible that the number of disease states found to correlate with mutations in glycosyltransferases, or other enzymes contributing to oligosaccharide synthesis, may increase significantly during the next several years. It is possible, for example, that a galactosyltransferase mediating proteoglycan synthesis is mutated in patients with Progeroid syndrome [29].

Significant variation of glycosyltransferase expression occurs during cellular differentiation as well as in the pathologic behaviour of various transformed cell types. For example, *N*-acetylglucosaminyltransferase-V (GnT-V) activity is highly induced in metastatic cells and upon neoplastic conversion of cells by various stimuli, including expression of *src* and *ras* oncogenes [30–31]. Thus, GnT-V may regulate the transformed and metastatic behaviour of these and other tumorigenic cells. Should GnT-V prove to be a proto-oncogene that can be activated to oncogene status by overexpression or mutation, increased activity of this glycosyltransferase may be a mechanism for the action of various kinases and G-proteins in the aetiology of certain cancers.

Cell lines have been isolated in tissue culture systems

that harbour mutations in several steps in the biosynthesis of *N*- and *O*-linked glycans and glycosaminoglycans [32–33]. Additionally, cell lines have been analysed in the presence of inhibitors of glycosylation [34–35]. The surprising finding in these studies is that perturbation of the glycosylation machinery has relatively little effect on the ability of the cell to grow and function in an *in vitro* tissue culture environment. This fact together with the apparent rarity of genetic diseases involving glycosylation defects have suggested to many glycobiologists that glycoconjugates could play essential roles in complex biological systems. For example, these structures may function in modifying cell–cell interactions that act to regulate morphogenic processes in embryogenesis. Experimentally-defined perturbations of glycosyltransferase genes may therefore provide the key to understanding the function of specific oligosaccharide moieties in ontogeny, physiology and disease etiology.

Transgenic models: news from ontogeny and physiology

To test such hypotheses it is necessary to generate controlled perturbations of glycosyltransferases *in vivo* and during ontogeny. There are several approaches in vertebrate animals, especially mice. Reagents which affect oligosaccharide structure (lectins, anti-carbohydrate antibodies, glycosidases, degradative enzymes acting on carbohydrates, etc.) could be applied to the developing embryo. However, such techniques in mammalian ontogenic approaches would be highly invasive, difficult to regulate and duplicate, and likely suffer from toxic side-effects. In studying the genetics of glycosyltransferase function *in vivo*, perhaps the most informative approach is obtainable by transgenesis.

Techniques to modify the genome of intact animals have advanced enormously. Experimentally-designed DNA constructs that are incorporated into somatic cells and the germline are defined as transgenes. Although initial experiments included the use of viral particles [36], the experimental constraints imposed by this method led to a simplified approach that relied upon micro-injection of solutions of purified DNA into one-cell embryos [37–38]. This is accomplished by obtaining embryos approximately 12 hours after fertilization, prior to fusion of male and female pronuclei, and injecting several picolitres of DNA solution containing several hundred copies of the purified gene construct. Integration of this DNA into chromosomes is random in position and may occur in approximately 25% of embryos injected. On average, 50% of injected embryos usually survive and these are implanted into pseudo-pregnant female mice (by virtue of mating with a vasectomized male) that then become surrogate mothers. The majority of implanted embryos survive and approximately 20% of mice born may be transgenic as defined by DNA analyses. Since transgene integration will generally occur prior to the first mitotic division of the embryo, all cells of transgenic mice commonly test positive for transgene sequences. This approach is applicable to dominant genetic studies of the biological effects of additional or ectopic gene expression, as exemplified below.

Transgenic expression of a viral sialic-acid-specific 9-*O*-acetylesterase in mice has yielded provocative results implying a crucial role for complex carbohydrates in early pre-implantation development. In this study an inhibition of the first mitotic division occurred [39]. Additionally, various organ abnormalities were observed when this sialylesterase was expressed in distinct tissues. Such control over expression is made possible by incorporating various tissue-specific regulatory elements that can restrict transgene expression to certain cell types. Reports that a later stage of pre-implantation development is regulated by specific oligosaccharide structures were derived from studies of morulae stage embryo compaction and blastocyst formation involving β1-4 galactosyltransferase and lactosaminoglycans [40–42]. It is worth noting that since the embryonic genome of the mouse is not significantly activated prior to the eight-cell stage, lethality before this would not be expected to reproduce the exact biological correlate of a genetic loss in glycoform production. Although lethality may subsequently occur, modifying the endogenous expression program of recently isolated glycosyltransferase genes will be important in providing crucial biological and physiological data. Moreover, while ectopic expression and overexpression of specific glycosyltransferase genes may reveal important functional attributes, transgenic mouse studies following homozygous inactivation of specific endogenous genes (null mutations) have led to some of the most valuable biological information gained in the context of whole organism physiology.

The generation of specific and experimentally-induced null mutations has previously been restricted to the realm of yeast geneticists. Those fortunate ones have gained remarkable advances in the biological understanding of gene function by such genotype–phenotype studies. This was possible since yeast preferentially incorporate exogenous DNA via homologous recombination. Although gene inactivation in mammalian cell lines can be achieved by some strategies, eg, the lectin-mediated selection of somatic cell line mutants [32], homologous recombination with exogenous DNA does not occur at nearly the frequency in mammalian cells as it does in yeast. Therefore, the incorporation of directed mutations into the germline of mammals awaited the derivation of pluripotent embryonic stem (ES) cells and methods to culture them *in vitro*.

Studies in the 1980s revealed that cells derived from the inner cell mass of pre-implantation mouse embryos, and appropriately cultured *in vitro*, could contribute to the germline of chimeric mice produced by injecting these ES cells back into pre-implantation embryos [43–44]. Moreover, genetic modifications to cultured ES cells could be achieved by classical gene transfer approaches. However,

the ability routinely to produce defined null mutations awaited development of the polymerase chain reaction (PCR). PCR allows geneticists to screen for adjacent positioning of exogenous and endogenous DNA following homologous recombination in mammalian ES and somatic cells, an event that may require analyses of several thousand cell clones representing distinct exogenous DNA integration events [45–47].

In the last few years, and for the first time, the door has been opened allowing mammalian geneticists to produce the most crucial of model systems *in vivo* – the null genotype. Recent advances in this technology, referred to as 'gene-targeting' or the production of 'gene knock-out' mice, have increased the efficiency of homologous recombination by the use of 'positive-negative' selection strategies and 'isogenic' DNA vectors [48–49]. The use of these approaches, especially with isogenic DNA, now often results in homologous recombination frequencies of greater than 1–10% of stable integrations screened. As is obvious from the scientific literature of recent years, the mouse has now more than ever become a crucial mammalian genetic model. Hence our understanding of mammalian ontogeny and physiology is undergoing a revolution at present. Moreover, such studies have recently produced new insights into the function of complex *N*-glycans.

Complex *N*-glycans: loss yields evidence for specific roles in mammalian ontogeny

UDP-*N*-acetylglucosamine: α-3-D mannoside β1-2-*N*-acetylglucosaminyltransferase I (GnT-I) is an essential and key enzyme in the synthesis of *N*-glycans. Mutant cell lines lacking this enzyme cannot convert high mannose *N*-glycans to complex or hybrid *N*-glycans yet can grow and function normally under *in vitro* tissue culture conditions [32, 50–53]. The recent cloning of the *Mgat-l* gene encoding mouse GnT-I by two laboratories [54–55] was prompted by the desire to inactivate GnT-I function in the intact mouse. Both laboratories have now succeeded in this endeavour [56 and P. Stanley, personal communication]. In both studies the homozygous *Mgat-l*$^{-}$/*Mgat-l*$^{-}$ genotype precluded the development of viable offspring thus establishing that complex *N*-glycans are crucial in ontogeny. Remarkably, embryogenesis in the absence of complex *N*-glycans proceeded into post-implantation and yielded specific and surprising phenotypes.

In the study by Metzler *et al.* [56], a normal Mendelian distribution of wild-type and mutant genotypes was observed throughout pre- and post-implantation development prior to 25% lethality. Unlike previous studies of oligosaccharide function, pre-implantation development was not affected. Moreover, mice that were heterozygous for *Mgat-l* inactivation were viable and phenotypically normal even though they had lost significant levels of GnT-I activity. Analyses of oligosaccharide structures confirming that *Mgat-l* null embryos were deficient in complex *N*-glycans and displayed increased levels of high-mannose *N*-glycans. In a particularly remarkable finding, the acquisition of left–right body plan asymmetry was randomized in the absence of complex *N*-glycans. As complex *N*-glycans therefore regulate signals affecting the emergence of left–right body plan asymmetry, it will be of interest to determine if a phylogenetic correlation can be found with organisms that harbour *Mgat-l* homologues and those that have evolved such asymmetry. Interestingly, in the presence of morphologic phenotypes, differentiated cells emerged unaffected in the absence of complex *N*-glycans. These observations support the hypothesis that cell differentiation, *per se*, is not regulated by complex *N*-glycans, but instead complex *N*-glycans appear to be required for specific morphogenic events that result from cell–cell interactions among differentiated cell types.

The above findings indicate the importance of GnT-I to normal mammalian developmental and morphogenic processes. However, those studies did not identify specific proteins that may have generated the observed phenotypes as a result of deficiency in complex *N*-glycans. This experimental limitation frequently occurs when studying enzyme mutants. For example, the loss of specific kinase and phosphatase genes, as a result of either natural sporadic events or gene-targeting experiments, generates alterations in phosphate levels on many proteins that are thus candidate substrates and potential phenotypic effectors [57–60]. Given our present-day cursory understanding of signal transduction events in whole organism physiology, future efforts are required to identify such 'downstream' effectors. Nevertheless, it is obvious that significant information is to be gained from analysing the biological consequences of such mutations. These experiments therefore permit a rational approach to both biological function and therapeutic target verification, as well as provide an important genetic reagent for future studies.

Inducible gene inactivation *in vivo*: a new approach for glycobiology

The production of embryonic lethal phenotypes, while obviously yielding valuable data, cannot disclose glycosyltransferase roles that may be relegated to later developmental stages, other cell types and adult function. Discerning the potential multiple roles of gene products normally expressed at various ontogenic stages, or among disparate cell types, has been intractable with gene targeting approaches to date. This experimental limitation could severely hinder efforts to understand the physiological relevance of the diverse and highly regulated expression patterns of glycosyltransferases *in vivo*. Fortunately, research efforts in this laboratory, as well as in others, have succeeded in developing a novel technology in which gene inactivation may be restricted to specific tissues and

cell types in an experimentally-defined and inducible manner.

The Cre site-specific DNA recombinase, a member of the integrase gene family, can efficiently eliminate mammalian chromosomal DNA flanked by 34-base pair recombinase target signals (loxP sites) *in vivo* depending upon tissues and cells that express the Cre transgene [61–62]. This is a two-tiered transgenic approach that retains a requirement for gene-targeting in ES cells, yet the design of the targeting vector is quite unique. LoxP sites and a selectable marker, usually the neomycin phosphotransferase (Neo) gene expression cassette, are placed in non-deleterious positions, such as introns and 3′ untranslated sequences. Additionally, a crucial exon of the gene to be targeted is positioned between the two loxP repeats. This modification to gene targeting vector design has not thus far affected homologous recombination frequency and at least four mouse genes have been so targeted in ES cells, including *Mgat-1* [63, and unpublished data]. Moreover, both pan-specific and tissue-specific gene inactivation models can be obtained from a single loxP-flanked gene-targeting event in ES cells since a sub-line of the parental loxP-flanked and gene-targeted ES cells can be derived following transient Cre expression; this then provides for the 'classical' systemic gene inactivation model [63]. For Cre-dependent and tissue-specific gene inactivation, two generations of progeny can result in mice that are homozygous for a loxP-flanked gene and transgenic for Cre. Since Cre transgene expression can be altered in an experimentally-regulated manner, glycosyltransferases may thus be inactivated in specific organs, cell types and at defined times during ontogeny. Such studies may provide the long-awaited Rosetta Stone to glycoconjugate function.

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